HPLC Columns for the Retention of Very Polar Molecules

By Paul Ross

INTRODUCTION

The requirement to retain and analyze polar molecules by HPLC is one that has grown steadily over the last few years, and has been the driving force behind the generation of a range of new stationary phases dedicated to this purpose. The new packings offer an alternative mechanism of interaction that takes place in addition to dispersive interactions generally associated with the more tradition alkyl silane type packings. Such interactions allow for increased retention and improved peak shape of analytes with polar functionality whether basic or acid in nature. Such packings are not only targeted at the separation of polar analytes but often encompass the application range of the traditional alkyl C18 packings also.

In this technical bulletin, we review columns made with several packings that have been tailored to go beyond the limitations of the traditional C18 packing materials. The columns that we review are listed as follows and are discussed individually throughout the bulletin:

- *AQUASIL C18 - Wettable reversed phase*
- *HyPURITY® ADVANCE - Imbedded polar group phase*
- *Hypercarb® – Porous Graphitic Carbon*
- *PRISM® - Imbedded polar group phase*
- *Fluofix® and Fluophase® - Perfluorinated phases*

Without exception, the phases outlined above achieve quite different chromatographic behavior to the more traditional type C18 packings in that they provide:

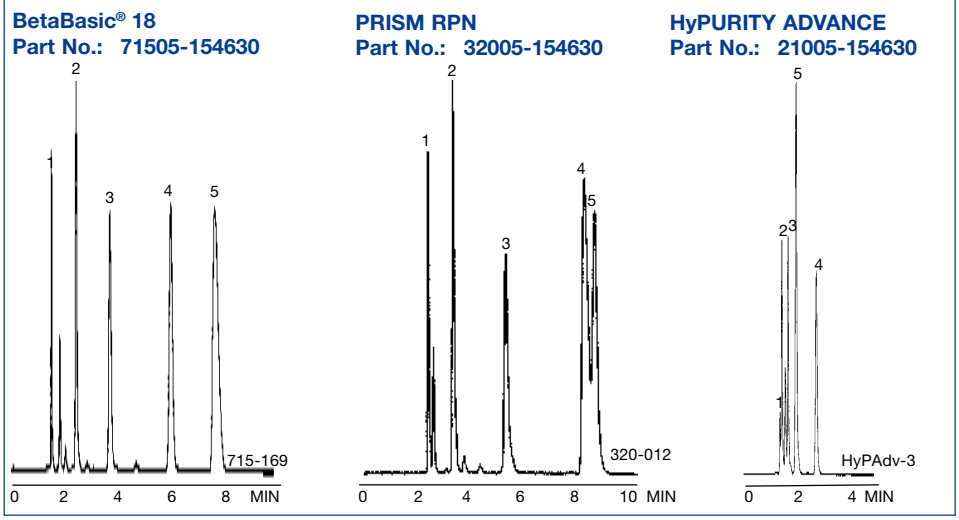
- *Alternative selectivity*
- *Increased retention of polar compounds*
- *The ability to run in highly aqueous mobile phase*
- *The ability to be used at reduced buffer concentrations*

Figure 1.

Retention of Basic Analytes on C18, PRISM and HyPURITY ADVANCE

Dimensions: 5µm, 150x4.6mm
Eluent: 10% ACN / 90% 0.05M KH_2PO_4, pH 3.5
Flow: 1.25 mL/min
Detector: UV @ 254

Sample:
1. Procainamide
2. Uracil
3. n-Acetylprocainamide
4. Caffeine
5. n-Propionylprocainamide



To achieve these chromatographic characteristics a certain degree of polar character is incorporated into the phase chemistry of the packing. Interactions that take place between the analyte and the stationary phase are then a mixture of dispersive interactions (as for traditional type C18 packings) and also dipole/hydrogen bonding interactions. The exception is porous graphite Hypercarb columns, where a quite unique mechanism of retention provides a surface that can retain both polar and non-polar analytes.

Figure 1 gives an indication of how two of the phases, the PRISM RPN column and the HyPURITY ADVANCE column, can give rise to alternative selectivity for the more polar basic compounds in a test mixture compared to C18. These phases are discussed in greater detail later in the bulletin.

The requirement to analyze compounds that contain increasing polar functionality is common to the pharmaceutical, environmental and food industries. This trend is driven by the need to identify not only drug compounds, pesticides or food substances but also the impurities and derivatives of the parent compounds that may be present. Often this requires investigating the effect these compounds have on the human body, and therefore may require detecting and measuring extremely small quantities of a compound in complex matrices. Alternatively, water samples may be taken from the environment where only trace levels of a pesticide may be found in our rivers and oceans. In both cases complex break down products in the environment or glycosilation of the compound in the body can lead to more complex and difficult HPLC analysis than first might have been envisaged for a new chemical.

Further to this, the coupling of Mass Spectrometry to an HPLC System has become common place in many laboratories. For specificity the compounds are analyzed in their ionic state. In this form molecules are at their most polar and we see again the drive towards analyzing increasingly polar molecules by HPLC.

The bulletin outlines the properties for each of the phases listed above that make them quite different to traditional C18 packings. Particular emphasis is placed on the following characteristics:

Selectivity

Packings that offer additional modes of interaction give rise to quite different retention behavior and selectivity. In general analytes with the greatest polar functionality will typically show greatest changes in selectivity and retention.

Highly Aqueous Mobile Phases – Increased Retention

The inclusion of polar functionality to the stationary phase also increases the wetting characteristics of the packing in highly aqueous mobile phases (100% aq). Several of the packings outlined in this bulletin can be run in 100% aqueous mobile phase conditions and show no tendency towards phase collapse or folding. Phase collapse is often seen for alkyl C18 packings where a small amount of organic solvent (1-5%) is generally required in the mobile phase to help wet the C18 surface and prevent phase collapse. See separate technical bulletin #TB99-01 for further explanation of phase collapse/folding.

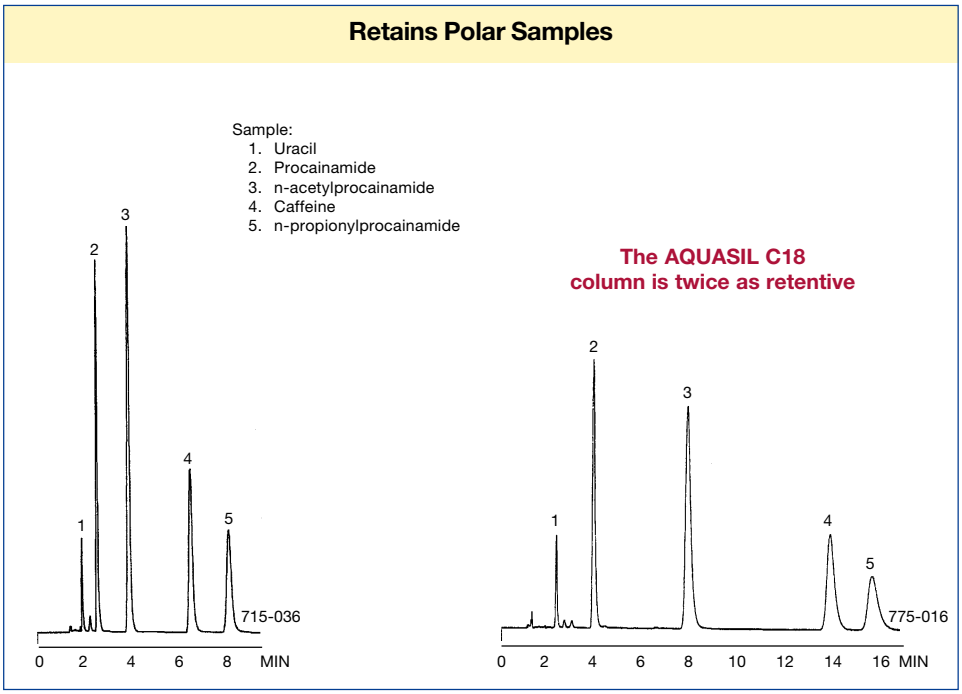
Reduced Buffer Concentrations – Increased MS Sensitivity

Several of the phases outlined in this report are also shown to maintain chromatographic performance when very low concentrations of buffers are used. Low buffer concentrations offer the reward of increased sensitivity for MS. This is an important consideration when trying to identify trace quantities of a drug compound or impurity that may normally disappear into the noise of the base line of the MS response.

Further Information

A brief review of typical questions that are often raised concerning these stationary phases is given in the following pages. For more in-depth information on each product please request the individual Product Bulletins (as highlighted for each product) from Thermo Hypersil-Keystone Technical Support.

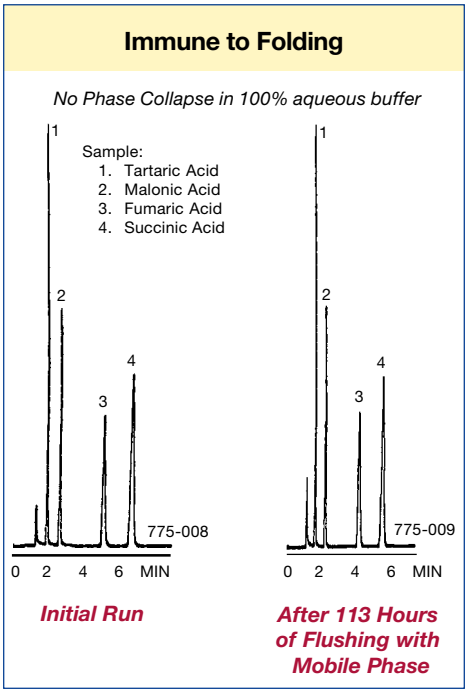
Figure 2.



BetaBasic 18, 5µm, 150x4.6mm
Part No.: 71505-154630
Eluent: 10% ACN / 90% 0.05M KH_2PO_4, pH 3.5
Flow: 1.25 mL/min
Detector: UV @ 254

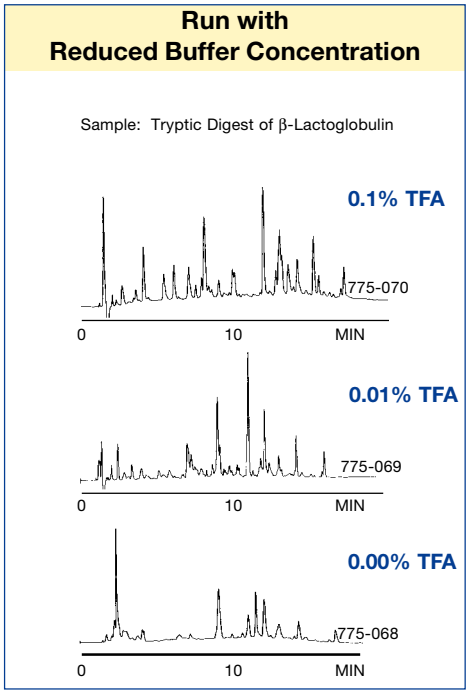
AQUASIL C18, 5µm, 150x4.6mm
Part No.: 77505-154630
Eluent: 10% ACN / 90% 0.05M KH_2PO_4, pH 3.5
Flow: 1.25 mL/min
Detector: UV @ 254

Figure 3.



AQUASIL C18, 5µm, 150x4.6mm
Part No.: 77505-154630
Eluent: 0.05M KH_2PO_4 +0.03M H_3PO_4
Flow: 1.25 mL/min
Detector: UV @ 210

Figure 4.



AQUASIL C18, 5µm, 150x4.6mm
Part No.: 77505-154630
Gradient: A: TFA in H_2O
B: TFA in ACN
10% B to 50% in 20 min, linear to 10% B at 21 min, hold to 30 min
Flow: 1.0 mL/min
Detector: UV @ 220

For more information, please request Product Bulletin 99-03

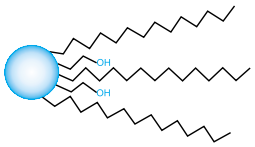
AQUASIL C18 Columns

How does retention of polar analytes compare with that of tradition C18 alkyl bonded phase?

The AQUASIL C18 phase was designed for the reversed phase separation of polar molecules. It has a high concentration of C18 groups as well as hydrophilic sites that help to provide retention of highly polar water soluble compounds (Figure 2), but offers the added benefit of nearly twice the retention of polar compounds when compared to BetaBasic® 18.

AQUASIL C18 retention is comparable to a traditional C18 when run in mobile phase with high organic.

AQUASIL C18 with polar functionality



Can I use the AQUASIL C18 in 100% aqueous mobile phase?

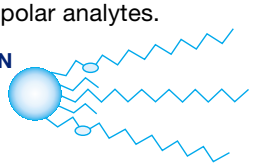
Yes! The extra polar character associated with AQUASIL C18 allows the use of mobile phases without any organic component, i.e. 100% aqueous (Figure3). Traditional C18 packings with high carbon loads require at least 3 to 5% organic component in the mobile phase to prevent phase collapse (folding). During this process, the retention and selectivity of the phase is lost and the column must be regenerated using a pure organic solvent wash. AQUASIL C18 is immune to this folding due to its unique polar functionality.

Can I run low pH applications with reduced buffer concentration?

When used in high concentrations, buffers (e.g. trifluoroacetic acid) can cause MS ion source supression and consequently can reduce sensitivity. The choice of column used is of key importance for LC/MS applications since the quality of the C18 packing and underlying silica can strongly influence the concentration of buffer required. In this example, we show that buffer concentration (TFA) can be reduced to zero concentration without loss in performance when using AQUASIL C18 (Figure 4). It is generally good practice to buffer your mobile phase, but with the AQUASIL C18 column, very low concentrations can be used.

PRISM® RP and PRISM RPN Columns

PRISM was developed to offer alternative selectivity to traditional alkyl C8 or C18 packings. It has unique chemistry that involves the incorporation of polar functional groups near the silica surface. These polar functional groups also form part of the alkyl chain that is responsible for the primary mode of interaction between the stationary phase and the analyte. Imbedded polar groups allow for a secondary or mixed mode type of interaction to take place, which in turn leads to a quite different retention behavior for polar analytes.



Are PRISM columns available in both endcapped and non-endcapped versions?

Yes. Both columns offer alternative selectivity and excellent peak shape for basic compounds. The non-endcapped PRISM RPN phase gives different selectivity for moderately polar and basic analytes such as tricyclic antidepressants (Figure 5).

The endcapped PRISM RP phase gives slightly different selectivity and offers improved peak shape for acidic analytes in particular.

Can the PRISM RP and PRISM RPN columns be run in 100% aqueous mobile phase?

Yes. In order to maximize retention of many very polar compounds, it is usual practice to reduce the percentage of the organic component in the mobile phase. Packings, such as PRISM RP and PRISM RPN, which contain imbedded polar groups near the surface of the silica, allow the organic component of the mobile phase to be reduced to zero, therefore maximizing the possibility of retention of highly solvated polar molecules (Figure 6).

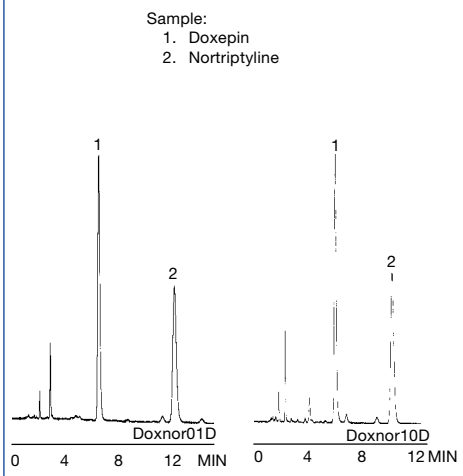
How does trifluoracetic acid (TFA) concentration affect resolution?

Figure 7 shows the separation of three simple Angiotensin peptides. The resolution is shown to improve significantly for peaks 1 & 2 as the concentration of TFA decreases to 0.01%.

Increasing the TFA concentration is generally thought to increase the hydrophobic properties of basic analytes when in ionic state. It does this by displacement of water molecules with TFA counterions. An increase in retention is observed as the concentration of TFA is increased but also a loss of resolution. Even at low concentrations of TFA, the polar PRISM packing can interact with the analyte to provide increased resolution. Changes in pH of the different concentrations can also affect resolution.

Figure 5.

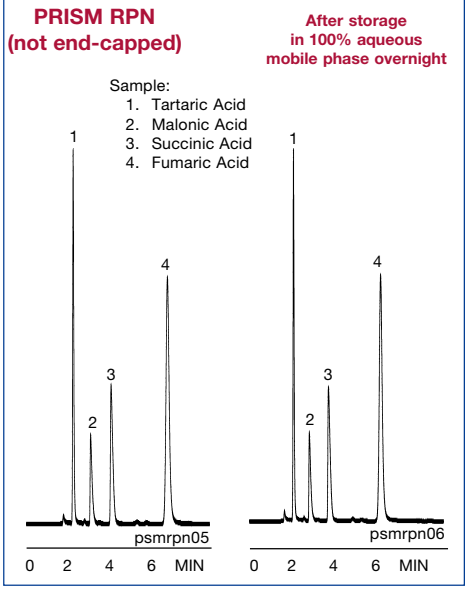
Antidepressants



Dimensions: 5µm, 150x4.6mm
Part No.: 32105-154630
Eluent: 25% ACN / 75% 0.05M
Flow: 1.25 mL/min
Detector: UV @ 254

Figure 6.

PRISM RPN Phase Immune to Folding



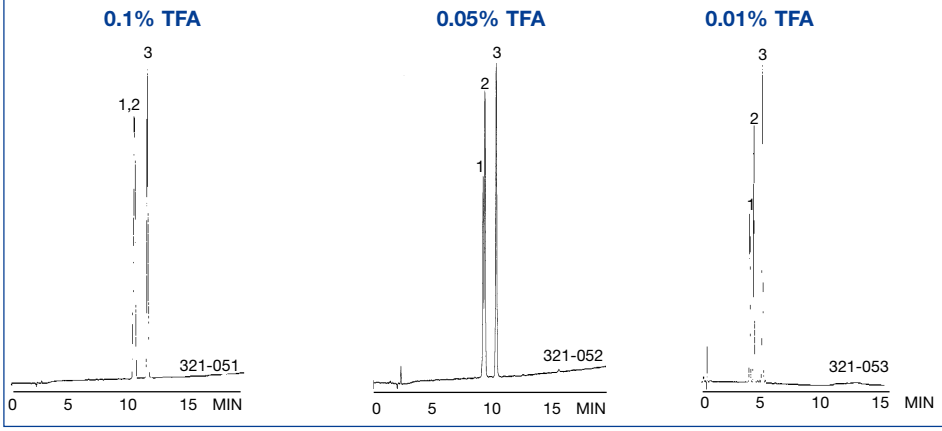
Dimensions: 5µm, 150x4.6mm
Part No.: 32005-154630
Eluent: 0.05M KH_2PO_4 +0.03M H_3PO_4
Flow: 1.0 mL/min
Detector: UV @ 210

Figure 7.

Effect of TFA Concentration on Resolution

PRISM RP, 5µm, 150x4.6mm
Part No.: 32105-154630
Gradient: A: TFA in H_2O
B: TFA in ACN
10% B to 50% in 20 min
Flow: 1.0 mL/min
Detector: UV @ 220

Sample:
1. Angiotensin III
2. Angiotensin II
3. Angiotensin I



For more information, please request Product Bulletin PB01-18.

Figure 8.

HyPURITY ADVANCE Retention Mechanisms

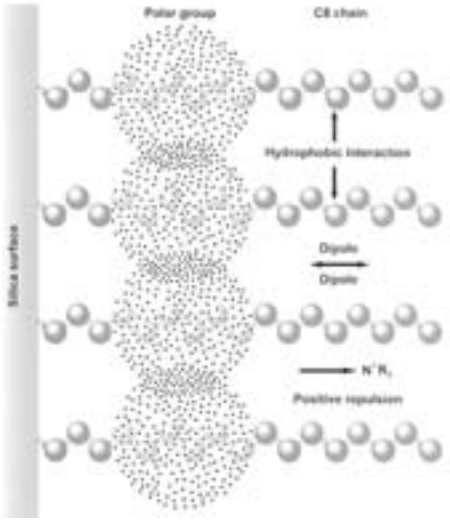
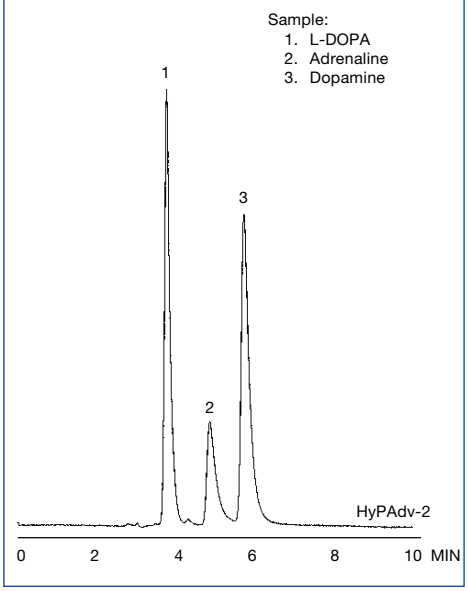


Figure 10.

Catecholamines in 100% Aqueous Mobile Phase



HyPURITY ADVANCE, 5µm, 250x4.6mm
Part No.: 21005-254630
Eluent: 100% 20mM K_2HPO_4, pH 7.5
Flow: 1.0 mL/min
Detector: UV @ 270
Temp: 25°C

Figure 9.

Alternative Selectivity

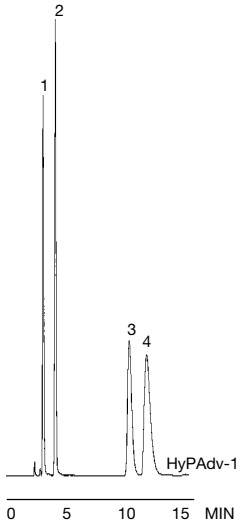
HyPURITY C18 Column
Part No.: 22105-154630

Sample:
1. Sulfathiazole

2. Sulfanethiazole

3. Sulfadimethoxine
4. Sulfaquinoxaline

HyPURITY ADVANCE Column
Part No.: 21005-154630



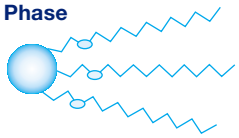
Dimensions: 150x4.6mm
Eluent: 72% Heptanesulfonic acid / 28% MeOH
Detector: UV @ 254

For more information, please request the HyPURITY Applications Booklet and HyPURITY Product Guide.

HyPURITY® ADVANCE Columns

HyPURITY ADVANCE was developed to offer alternative selectivity to the traditional alkyl C8 or C18 packings. It has unique chemistry that involves the incorporation of polar functional groups (different chemistry than PRISM® phases) near the silica surface. An alkyl C8 group is responsible for the primary mode of interaction between the stationary phase and the analyte. The imbedded polar groups allow a secondary or mixed mode type of interaction to take place, which leads to quite a different retention behavior and improved peak shape for many of the more polar analytes.

HyPURITY ADVANCE Phase
Polar embedded group with C8 chemistry



Should I expect increased or decreased retention of my polar analytes?

Basic compounds generally show reduced retention on HyPURITY ADVANCE columns, especially at pH 2-5 where the imbedded polar group carries a positive charge and can repel approaching similarly charged groups (Figure 8).

Acidic compounds conversely are retained slightly longer. Both effects contribute to quite different retention behavior compared to traditional C18 silicas with the added advantage that excellent peak shapes are obtained for acidic, basic and neutral compounds. Where ionization of the analyte has been suppressed, interaction takes place via dispersive interactions with the C8 group, and also by dipole-dipole interactions between polar groups on the stationary phase and the polar groups on the analyte. This gives rise once again to alternative selectivity (Figure 9).

Can I run HyPURITY ADVANCE columns in 100% aqueous mobile phase?

Yes. The HyPURITY ADVANCE phase shows a complete absence of folding or phase collapse when run in 100% aqueous conditions.

Figure 10 shows an example where the HyPURITY ADVANCE column has been used to analyze several polar catecholamines. The mobile phase consists of 20mM phosphate buffer, pH 7.5. The analysis is run in complete absence of any organic modifier.

Are there other applications available that have been run on the HyPURITY ADVANCE column?

Yes. Please request the HyPURITY Applications Booklet and also the HyPURITY ADVANCE Product Guide for further information on selectivity, stability, compatibility with MS and speed of analysis.

Fluorinated Phase Columns

Fluorinated stationary phases offer a new approach to stationary phase selectivity. Fluorine atoms with their highly electronegative character offer alternative molecular interactions by which polar analyte retention can take place. Three different packings are available; see Figure 11 to compare selectivity.

Does the fluorine chemistry give rise to alternative chromatographic selectivity?

The fluorine chemistry often behaves in a similar manner to a traditional alkyl bonded packing. Where an analyte has some polar functionality, fluorinated packings can often give quite different selectivity. This is particularly the case for fluorinated or chlorinated compounds. Fluofix and Fluophase columns have also been shown to give excellent results on non-halogenated compounds such as lipids, surfactants, taxanes, catechins and many other polar compounds with carboxyl or nitro groups.

Figure 11 shows how improved resolution can be obtained using Fluophase RP and Fluofix when compared to analysis of the same compounds using BetaBasic 18. Similar reports have been observed for isomers or compounds closely related in structure.

Can I use 100% aqueous conditions with fluorinated phases?

Resistance to folding is observed for both the Fluofix and Fluophase RP. Fluophase PFP has shown some tendency to fold under 100% aqueous conditions and for this reason it is recommended that at least 5% of the mobile phase composition should contain organic solvent (Figure 12).

What different fluorinated phases are available?

Fluofix 120E is a fluorinated branched-chain hexyl phase on 120Å silica. Fluophase RP and Fluophase WP are fluorinated straight-chain hexyl phases on 100Å and 300Å silica, respectively. Fluophase PFP is a pentafluorylphenyl phase on 100Å silica.

Figure 11.

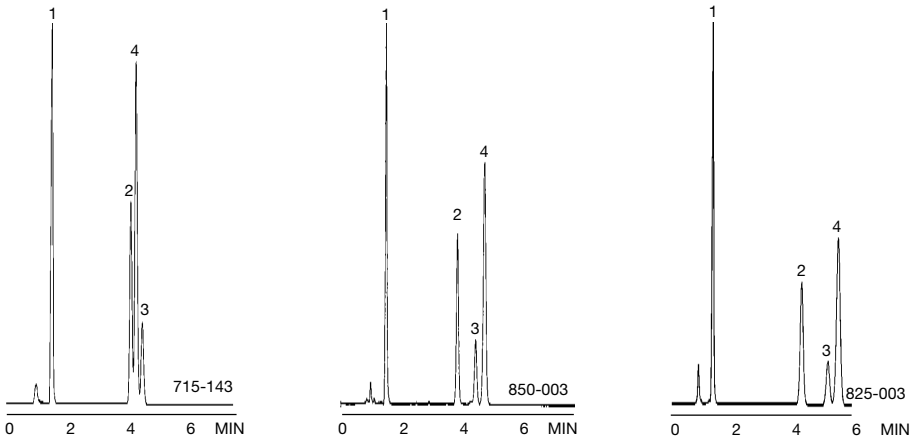
Improved Selectivity of Fluorinated Compounds

Sample:
1. Uracil
2. 1,3 Difluorobenzene
3. 1,3,5 Trifluorobenzene
4. 1,2,4,5 Tetrafluorobenzene

BetaBasic®18 Column
Part No.: 71505-154630

Fluofix ®120E Column
Part No.: 21131

Fluophase® RP Column
Part No.: 82505-154630



Dimensions: 5µm, 150x4.6mm
Eluent: 60% ACN / 40% H_2O
Flow: 1.0 mL/min
Detector: UV @ 254

Fluofix and Fluophase Perfluorinated Phases

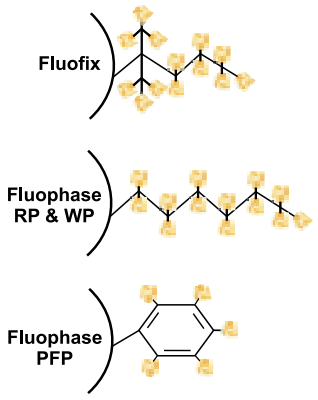
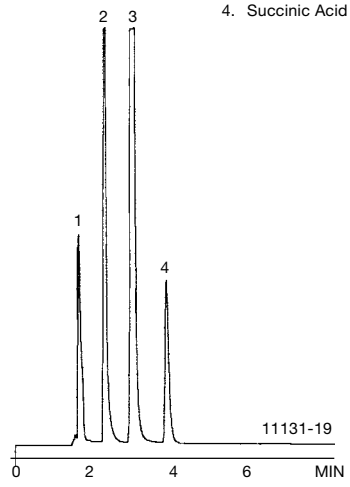


Figure 12.

Resistant to Folding

Sample:
1. Tartaric Acid
2. Malonic Acid
3. Fumaric Acid
4. Succinic Acid

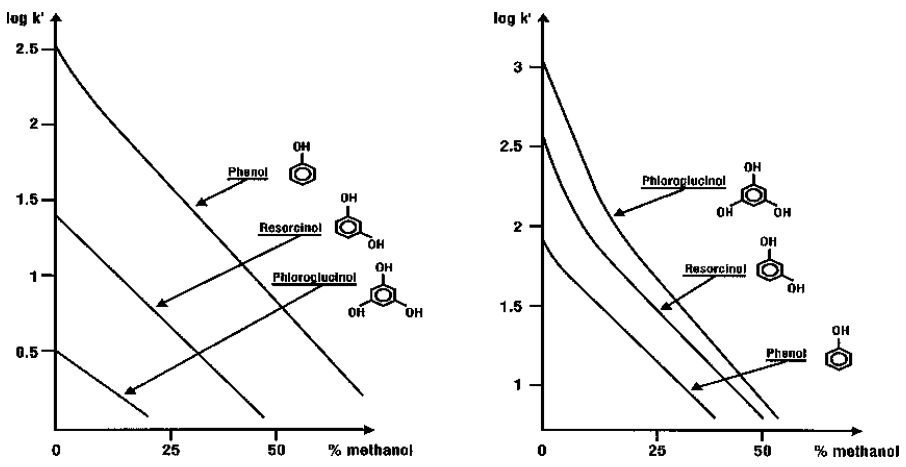


Fluofix 120E, 150x4.6mm
Part No.: 21131
Eluent: 0.02M KH_2PO_4, pH 3
Flow: 1.0 mL/min
Detector: UV @ 210

For more information, please request Product Bulletin PB01-11.

Figure 13.

Polar Retention Effect on Hypercarb®

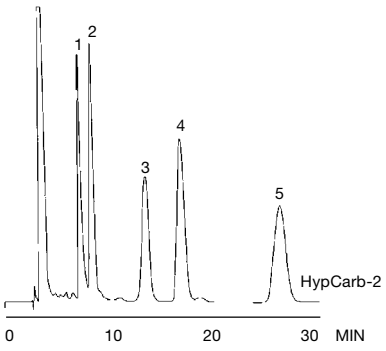


Courtesy of V.Cocquart & M.C. Hennion, J.Chrom, 1992

Figure 14.

Polar Pharmaceutical Compounds

Sample: Morphine and Metabolites
1. Normorphine
2. Morphine-3-glucuronide
3. Morphine-6-glucuronide
4. Morphine
5. Codeine

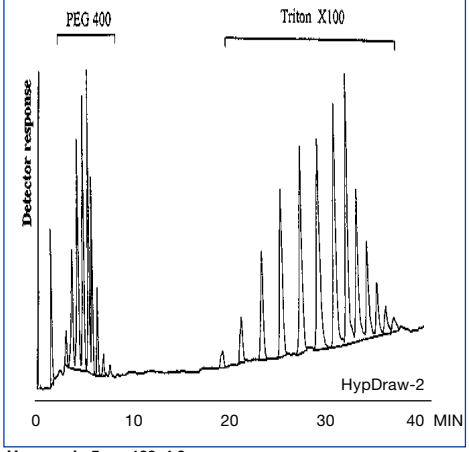


Hypercarb, 5µm, 100x4.6mm
Part No.: 35005-104630
Eluent: 60% MeOH / 40% Ammonium Acetate, pH 9
Flow: 1.0 mL/min
Detector: UV @ 220
Courtesy of Wan, Shaw, Davies and Barrett, Nottingham Univ.

Figure 15.

Polyethoxylated Alcohols and Phenols

Non-Ionic Surfactants



Hypercarb, 5µm, 100x4.6mm
Part No.: 35005-104630
Gradient: A: H_2O B: ACN C: Dichloromethane
From 20% A - 100% B during 15 min then 0% C to 80% C in 25 min
Flow: 1.0 mL/min
Detector: UV @ 220
Courtesy P.Chambault et al. J.Chromatogr A 797 (1998)

For more information, please request the Hypercarb Product Guide.

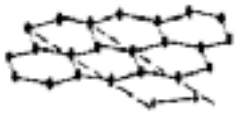
Hypercarb – Porous Graphitic Carbon Columns

Porous graphitic carbon has unique properties as a stationary phase and now often provides solutions to what might be considered 'problem HPLC separations'. Two such problem areas are:

(1) the retention and separation of very polar analytes not normally retained on C18 packings.

(2) the separation of structurally similar compounds, such as geometric isomers and diastereomers , not always separated on C18 silica.

Hypercarb porous graphitic carbon



Compounds of increasing polarity are retained more strongly on Hypercarb

The unique mechanism by which analytes are retained at the graphite surface gives rise to many new and attractive opportunities for the retention of polar molecules.

Figure 13 shows how retention behavior changes for phenol and some polyhydroxybenzenes versus percent organic modifier in the mobile phase. Note how the retention order differs for both Hypercarb and Hamilton PRP:

Hypercarb: 1,3,5 trihydroxybenzene >1,2 dihydroxybenzene > phenol.

PRP-1: Phenol>1,2 dihydroxybenzene > 1,3,5 trihydroxybenzene

(Note: under these conditions the two polyhydroxylbenzene analytes are typically not retained on C18 packings.)

Figure 14 shows retention of several very polar pharmaceutical compounds not normally retained on alkyl C18 silica.

What other benefits does Hypercarb offer over traditional alkyl bonded silica packings?

- *Enhanced selectivity for closely related compounds*
- *Retain highly polar compounds very strongly*
- *Stable across the pH range 1-14*
- *Can be used with a wide range of solvents from 100% aqueous to 100% hexane or methylene chloride*

The flexibility of solvent choice is demonstrated in Figure 15 where polyethoxylated alcohols and phenols are separated on a single Hypercarb column. It is common practice to have to use two columns for this analysis – a C18 for the reverse phase separation of the more polar analytes and a normal phase column for the very hydrophobic analytes.